Cerebral Anoxia: Protein Metabolism During Recovery in In Vitro Model

BY TAKEHIKO YANAGIHARA, M.D.

Abstract: Cerebral Anoxia: Protein Metabolism During Recovery in In Vitro Model

Amino acid incorporation into proteins of rabbit brain slices was studied during the recovery stage after various anoxic periods in an in vitro experimental model. There was a distinct difference between anoxic periods up to five minutes and those beyond ten minutes. In the former, inhibition of amino acid incorporation was of lesser degree and was reversible. On the other hand, more than 50% inhibition was observed following anoxic periods of 30 minutes and this inhibition was irreversible. Amino acid incorporation into proteins was more sensitive to anoxia than was amino acid transport into brain slices.

Additional Key Words: brain slices amino acid incorporation amino acid uptake

Biochemical aspects of cerebral anoxia or ischemia often have been studied in relation to cerebrovascular disease or cardiac arrest, and the major interest has been focused on the anoxic period. However, from the therapeutic point of view, it would be important to understand the tissue reaction immediately after the episode of anoxia. The purpose of the present investigation was to establish an in vitro experimental model suitable for such studies. Although an in vitro system has various disadvantages compared to the in vivo system, it offers an easier approach to detailed biochemical analysis with relatively good reproducibility and certain advantages for investigation of precursor incorporation into macromolecules. It also provides adequate amounts of homogeneous tissue for further biochemical investigations at the cellular or subcellular level. This paper describes amino acid incorporation into proteins of brain slices and shows a relationship between duration of anoxia and reversibility of inhibition of protein metabolism.

Methods

Albino rabbits (2.5 to 3.0 kg) were used throughout the investigation. Each animal was anesthetized by intravenous injection of pentobarbital (60 mg/kg) and perfused with ice-cold Ringer’s solution through a cannula inserted in the left ventricle of the heart via thoracotomy. The brain was quickly removed and placed at 4°C. The cerebral hemispheres were sliced (0.4 mm thick) with a McIlwain tissue chopper. The slices were distributed into three groups of approximately the same amount and placed in an incubation medium which has been used extensively for investigation of brain metabolism in vitro.1 2

Ten milliliters of incubation medium of the following composition (final pH 7.4) was used for approximately 1.5 gm of brain tissue in 50-ml Erlenmeyer flasks: Tris-HCl buffer (pH 7.4), 35mM; sodium phosphate buffer (pH 7.4), 5mM; NaCl, 100mM; KCl, 5mM; MgCl2, 2.5mM; ATP, 2.0mM; and glucose, 20mM. The first flask (group 1) was bubbled and flushed with 100% oxygen; and the second (group 2) and the third (group 3) flasks were bubbled and flushed with 100% nitrogen before and after the tissue samples were added. All flasks were tightly sealed and...
incubated at 37°C with constant shaking. Incubation was carried out for three, five, seven, ten, 20, and 30 minutes.

At the end of incubation, tissue from each flask was quickly recovered by filtration, further divided into four portions, and placed in separate 25-ml flasks containing 5 ml of ice-chilled incubation medium of the same composition but with addition of L-leucine-4,5-3H (Amersham/Searle or New England Nuclear; prepared to specific activity of 100 mCi/m mole and neutralized) at a concentration of 5 Ci/ml medium. Each flask for groups 1 and 2 was thoroughly flushed with 100% oxygen; for group 3 each was flushed with 100% nitrogen. These flasks were then incubated at 37°C under constant shaking; one flask from each group was removed from the water bath at 15, 30, 45, and 60 minutes.

In another series of experiments for groups 1 and 2, incubation was carried out in three stages with the same medium as before. For the first stage, brain slices were incubated in 50-ml flasks containing 5 ml of ice-chilled incubation medium under an oxygen atmosphere for 30 minutes; then they were transferred to another 50-ml flask. The second stage was further flushed with oxygen for group 1 and with nitrogen for group 2. Incubation for this stage was carried out for either five or 20 minutes. In the third stage, all flasks were incubated under oxygen, and tissue samples were obtained at 15, 30, 45, and 60 minutes.

At the end of each incubation, one or two brain slices were taken, briefly rinsed in 0.32M sucrose in 10mM Tris-HCl buffer (pH 7.4), blotted briefly on filter paper, and weighed. These slices were then homogenized in 1.0 ml of cold 5% trichloroacetic acid (TCA), and the supernate was obtained by centrifugation. The rest of each tissue sample was diluted to 40 ml with 0.32M sucrose solution described above and recovered by brief centrifugation. The pellet was homogenized in cold 5% TCA. The resulting precipitate was recovered by centrifugation, washed three times with cold 5% TCA (the third wash heated for 15 minutes at 90°C), and extracted with alcohol-ether (1:1) and ether. The dried sample was dissolved in 1N sodium hydroxide and an aliquot was taken for determination of protein content according to Lowry and co-workers. Another aliquot was added to a plastic counting vial, solubilized with Soluene-100 (Packard Instrument Co.), and counted in toluene-based scintillation solution in a Packard Model 3375 Tri-Carb liquid scintillation spectrometer. The channel ratio was used for quench correction and the final result (specific radioactivity) was expressed as disintegrations per minute per milligram of protein (DPM/mg protein).

For determination of TCA-soluble radioactivity, an aliquot of the TCA supernatant obtained from one or two brain slices was added to a glass counting vial, and the radioactivity was determined in Insta-Gel (Packard Instrument Co.) in

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td><strong>Leucine Incorporation Into Proteins of Brain Slices After Various Periods of Anoxia</strong></td>
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<td>Period of anoxia (min)</td>
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*Incubation sequences: group 1, O2-O2; group 2, N2-O2; group 3, N2-N2.
†Results are expressed as mean ± SE; number of experiments shown in parentheses.
Leucine incorporation in brain slices after various anoxic periods. Specific radioactivity (DPM/mg protein) in group 2 is expressed as percentage of that of group 1; results are shown as means ± SE with number of experiments as shown in table 1. Incorporation periods: 15 minutes, dark bar; 30 minutes, white bar; 45 minutes, striped bar; and 60 minutes, stippled bar.

Results

For group 1, leucine incorporation into proteins was approximately linear during the second incubation after various periods of the first incubation (table 1). This indicates the adequacy of the incubation system for the present study. Since the amount of radioisotope added for leucine incorporation varied to some extent in each experiment, there was some variation among experiments.

Although leucine incorporation was carried out under an oxygen atmosphere, there were various degrees of inhibition in leucine incorporation in group 2, which became more prominent at longer periods of anoxia during the first incubation. Although very markedly inhibited, group 3 showed radioactivities approximately 10% of those of group 1. This could be due to incomplete saturation of the incubation medium with nitrogen during the second incubation.

If the incorporated radioactivity in group 2, which is the main focus of the present experimental plan, is expressed as a percentage of the value in group 1 for each experiment and at each time interval, the effect of various anoxic periods can be seen more clearly (figure). There was a progressive increase in degree of inhibition in leucine incorporation into proteins as the anoxic period increased from three to 30 minutes, ranging from 25% to more than 50%. Inhibition became more apparent after anoxic periods beyond ten minutes. For each anoxic period, there was gradual recovery of leucine incorporation with increasing duration of incorporation (aerobic incubation) but, in general, recovery became more pronounced after aerobic incubation for 30 minutes. The values at 45 and 60 minutes reached 90% or more after anoxic periods of three or five minutes but only up to 60% for anoxic periods of 30 minutes. Since the normal variation (standard deviation) between two paired experiments under aerobic condition ranged from 10% to 15% (unpublished data), the recovery after anoxic periods of three or five minutes can be considered complete. Thus, there was a distinct difference between anoxia for three and five minutes, in which inhibition was reversible, and that of ten to 30 minutes, in which inhibition was irreversible up to a recovery time of 60 minutes. The data for an anoxic period of seven minutes appeared to be intermediate.

Table 2 shows the extent of leucine incorporation, after anoxic periods of five and 20 minutes, when brain slices were preincubated under aerobic condition. Comparison with corresponding data at five and 20 minutes (in the figure) suggests that inhibition at incorporation periods of 15 and 30 minutes was less, particularly for 20 minutes of anoxia. The extent of inhibition at 60 minutes was similar to that shown in the figure for anoxic periods of five or 20 minutes and no change was observed for reversibility of inhibition.

<table>
<thead>
<tr>
<th>Incorporation period (min)</th>
<th>5 min</th>
<th>10 min</th>
<th>20 min</th>
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<tr>
<td>15</td>
<td>76.2 ± 4.3</td>
<td>62.2 ± 5.0</td>
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<td>30</td>
<td>80.1 ± 2.3</td>
<td>67.6 ± 3.9</td>
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<td>45</td>
<td>82.9 ± 2.5</td>
<td>66.7 ± 4.3</td>
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<td>60</td>
<td>88.4 ± 5.2</td>
<td>69.6 ± 5.0</td>
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*Results are expressed as mean ± SE of percentages of control-specific radioactivities (DPM/mg protein) from four experiments.
The TCA-soluble radioactivity (leucine-
$^3$H uptake) is shown in table 3. The results in
group 1 suggest some tendency of higher
uptake after longer first aerobic incubation
periods but, on the other hand, there was a
trend toward progressive decrease in leucine
uptake as the incorporation time increased
during the second incubation period. There was
no obvious difference between groups 1 and 2
at any time period. The leucine uptake in group
3, under anaerobic conditions, was lower than
in group 1 or 2, but often the value came close
to that for aerobic incubation. This fact and
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to that for aerobic incubation. This fact and
the high variance of data from group 3 will be
discussed below.

**Discussion**

A number of experimental models of cerebral
anoxia and ischemia have been based on
inhalation of nitrogen, decapitation, or
closure of main arteries; these cause generalized
anoxic or ischemic lesions. For production of a
localization, which is the case in the
majority of instances of cerebrovascular
disease, a combination of carotid ligation and
exposure to nitrogen atmosphere or occlusion of
a major intracranial artery has been used.

Each of these models has advantages and
disadvantages.

The present in vitro model was designed
(1) to define the irreversibility of the anoxic
reaction from the biochemical standpoint,
particularly at the macromolecular level; (2)
to elucidate metabolic requirements, during
recovery, which would be important from the
therapeutic point of view; and (3) to produce
a large sample of homogeneous tissue which
would make it possible to characterize further
the reversible and irreversible processes by
investigation of macromolecular metabolism at
the cellular and subcellular levels.

A major question in regard to the validity
of the present model is the period (15 to 20
minutes) of anoxia which inevitably occurs
from the time of intracardiac perfusion to the
initiation of the first incubation. It is possible
that anoxic change during this period may
jeopardize the subsequent metabolic study.
However, this possibility can be minimized for
the following reasons. McLlwain stated that
the level of adenosine triphosphate (ATP) in
the brain slices was restored to 90% of initial
level by ten minutes after initiation of aerobic
respiration and this level could be maintained

### TABLE 3

<table>
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<tr>
<th>Brain Slices</th>
<th>TCA-soluble radioactivity (DPM/mg wet tissue)</th>
<th>15 min</th>
<th>20 min</th>
<th>30 min</th>
<th>45 min</th>
<th>60 min</th>
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- **Discussion**
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### TABLE 3

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<th>Leucine Uptake Into Brain Slices After Various Periods of Anoxia</th>
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*Incubation sequences: group 1, O₂-O₂; group 2, N₂-O₂; group 3, N₂-N₂.
†Results are expressed as mean ± SE; number of experiments shown in parentheses; no SE was calculated if results were based on two experiments.*
CEREBRAL ANOXIA

at least for two hours in the in vitro system. His statement is supported by unpublished data from this laboratory and by other data. Since ATP is one of the substances which disappears rapidly in cerebral anoxia or ischemia, the present experimental conditions should be satisfactory from this standpoint.

In the present study, the brain was rapidly perfused with ice-cold Ringer’s solution and then kept at 4°C until the initiation of incubation. This condition is similar to the profound hypothermia with circulatory arrest which has been used for neurological and cardiac surgery, and these patients recover without neurological deficits. Indeed, Kramer and co-workers demonstrated rapid return of brain ATP levels after profound hypothermia and subsequent circulatory arrest for 30 minutes. To ensure this aspect, one series of experiments in the present investigation was undertaken in which brain slices were maintained with aerobic respiration for 30 minutes prior to exposure to anaerobic atmosphere. There was no significant change in inhibition of leucine incorporation during the recovery stage, particularly in respect to reversibility of inhibition.

Although this three-stage experiment may prove to be more ideal in the future for other metabolic criteria, the two-stage experiment is more desirable for the subsequent experimental procedures. The principle of the in vitro experimental model described in this report appears feasible for investigation of certain biochemical aspects of cerebral anoxia, although the influence of preexisting anoxia may still remain; some modification of the experimental procedure may solve this problem in the future. Particularly for this reason, the critical time period (five minutes) for irreversibility of leucine incorporation should not be considered as representing critical duration of anoxia in vivo, although this duration has been cited in various species of animals.

Protein metabolism has been investigated sporadically in ischemic-hypoxic brain and hypoxic brain. In all these studies, a decrease of amino acid incorporation was demonstrated to various degrees. Blomstrand studied protein metabolism by using cellular and subcellular fractionation techniques and demonstrated inhibition of amino acid incorporation at the earlier stage under moderate hypoxia but an increase after prolonged exposure. The present data, particularly the aspect of reversibility, cannot be compared with his data because of differences in oxygen tension and exposure, time. Amino acid incorporation into protein is energy-dependent. Since it is known that ATP decreases during an anoxic episode, the question should be raised as to whether there is close correlation between the inhibition of leucine incorporation observed during the recovery stage and the tissue level of ATP. Although a preliminary study from this laboratory showed a significant difference of ATP level during anoxia, further investigation is necessary before a conclusion is drawn. In the present study, ample ATP and glucose were added to the incubation medium so that only the effect of oxygen level could be observed.

Active transport of amino acid into brain slices is also energy-dependent, although this is variable among different classes of amino acids; L-leucine appears to have a lesser degree of dependency on ATP. In the present study, no difference was observed between groups 1 and 2 once they were placed in the aerobic condition in an incubation medium with sufficient energy source. A surprising finding was the high leucine uptake in group 3 under anaerobic conditions. Guroff and associates demonstrated that brain slices have little ability for active transport of L-tyrosine under anaerobic conditions. Even considering the variation in the amount of leucine-$^3$H added in each experiment, the radioactivity per unit tissue weight is higher than the theoretical radioactivity of the incubation medium, thus indicating active transport of L-leucine in group 3. Since the amount of glucose in the incubation medium was very high, it is unlikely that so much leucine was converted to carbohydrate through the route of acetoacetate. Although this high TCA-soluble radioactivity might be due to a difference in class of amino acid, it is also possible that the small volume of oxygen remaining in the flask was the determining factor since removal of oxygen from the second incubation medium was not complete.

Comparison of protein radioactivity and TCA-soluble radioactivity in groups 2 and 3 indicates that amino acid incorporation into proteins is more sensitive to anoxia than is active transport of amino acid into brain.
Despite the high sensitivity of protein metabolism to anoxia, the inhibitory process can be reversible within a certain time interval, and this would offer an opportunity for investigation of the reactions of functionally and structurally important macromolecules of the central nervous system after anoxia.

Summary
Amino acid incorporation into proteins of rabbit brain slices was studied during the recovery stage after various anoxic periods in an in vitro experimental model. There was a distinct difference between anoxic periods up to five minutes and those beyond ten minutes. In the former, inhibition of amino acid incorporation was of lesser degree and was reversible. On the other hand, more than 50% inhibition was observed following anoxic periods of 30 minutes and this inhibition was irreversible. Amino acid incorporation into proteins was more sensitive to anoxia than was amino acid transport into brain slices. Advantages and disadvantages and future applicability of the in vitro experimental model were discussed.

Acknowledgments
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References
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